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MEMBRANE PROPERTIES AND LIPID PEROXIDATION IN FOOD RESTRICTED ANIMALS

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ABSTRACT

Food restriction (FR) is a well-recognized method of extending mean and maximum longevity of rodents, but the mode of its action remains to be uncovered. This article reviews the effect of FR on the physical-chemical properties and lipid peroxidizability of cellular membranes. FR prevents the age-dependent increase in microviscosity and peroxidizability of cellular membranes. It has been suggested that a decrease in the body temperature occurring in undernourished animals may play a fundamental role in the process. Indeed, the lowering of average body temperature occurring in FR animals may induce a modification in membrane lipid composition, stimulating the cells to counteract the rigidifying effect of lower temperature. Thus, membranes are maintained in a proper functional state by a mechanism similar to that found in poikilotherm animals.

INTRODUCTION

For over half a century, reduced feeding has been known to extend the life span of laboratory rodents (1). Since then, many independent studies have confirmed this finding (2-5). The food restricted (FR) model received particular attention during the last two decades, probably because dietary restriction's mode of action contributed to a better understanding of the basic mechanism(s) of aging. Yet, the mechanism by which undernutrition decelerates the rate of aging remains unclear. Nevertheless, we do know that the widespread impact of FR on animal physiology suggests an involvement of basic age-dependent modifications.

The free radical theory of aging proposed by Harman (6) hypothesized that aging results from macromolecular damage caused by free radicals generated during the course of normal metabolism. The role of membrane molecules was originally emphasized, with changes in their physical-chemical properties found to occur during aging in cells of human and animal origins (7-11). These changes were found to affect a wide variety of fundamental and age-dependent cellular processes, including ion transport (12,13), enzyme regulation activity (14,15), and signal recognition and transduction (16). Taking these findings into account, FR was hypothesized to exert its positive effect on the life span by protecting the physiological properties of cellular membranes against age-dependent modifications (17).

The analysis of cellular membranes properties was considered important for another reason. Some data presented in the literature suggested that the daily average body temperature was lower in undernourished rodents than in their ad libitum (AL) fed counterparts (18-20). Lane et al. (21) reported that calorie restriction also lowered body temperature in rhesus monkeys, consistent with earlier results on rats and mice.

There is no doubt that temperature greatly influences the physical-chemical properties of cellular membranes, in vitro, and that lower temperature is associated with an increased microviscosity of lipid domains. Therefore, FR animals, having a decreased average body temperature, should have more rigid membranes than their corresponding, age-matched AL-fed counterparts. However, this should result in the precocious aging of animals on restricted diets, which would go against the known "anti-aging" effect of FR. Moreover, because lipid peroxidation is known as one of the major events influencing membrane microviscosity (22-26), the peroxidizability of cellular membranes was also investigated by using either classical methods or with the aid of a new physiological model, the measurement of mitochondrial membrane potential. The present paper presents a critical review of current problems with membrane microviscosity and lipid peroxidation in FR animals.

Studies on Membrane Microviscosity

In order to solve the apparent paradox between the FR-induced lower body temperature and normal membrane function, several experiments were performed to compare the physical-chemical properties of membranes during aging between control and FR animals. The first experiments, in which the membrane properties were measured taking into account body temperature, were performed using female Wistar rats. FR was applied by

feeding the animals on an every-other-day schedule (EOD) with the same laboratory chow given to the AL control group. The treatment was started at the age of 3.5 months. Consistent with the expectation, the rats fed on an EOD schedule consumed less food per day than their age-matched, AL-fed counterparts. The difference was 21-22% decrease in food intake (27), and was sufficient to increase the mean, median, and maximum life spans of the undernourished rats (17).

Fluorescence polarization of the lipid probe 1,6-diphenyl-1,3,5-hexatriene (DPH) was determined in lymphocyte plasma membranes (17). The body temperature of EOD rats was not measured at the beginning of the experiment, but the measurement of microviscosity at three different temperatures allowed for an estimation of the eventual functional, in vivo, difference. As shown in Table 1, an age-dependent increase in microviscosity was observed at all temperatures, and when the results at the same temperature were compared, membranes of the EOD-fed animals were less viscous than those of young, AL-fed animals. It is also noteworthy that the lymphocyte plasma membrane of old EOD animals at 35°C was more fluid than that of old, AL animals measured at 37°C.

Table 1. Membrane microviscosity of lymphocytes (η)

Temperature (°C)	*Young*	*Old AL*	*Old EOD*
35	2.19±0.02	2.41±0.02	2.04±0.02
37	2.03±0.02	2.24±0.02	1.90±0.01

(Data in table are calculated from the figure reported by Pieri et al. (17), with the kind permission from Elsevier Science Ireland Ltd., Bay 15K Shannon Industrial Estate, Co. Clare, Ireland.)

In parallel experiments, the properties of liver plasma membrane was investigated using two probes, the DPH as before, and 5'-nucleotidase activity (28), a glycoprotein enzyme located primarily in the plasma membrane. It is an ectoenzyme (i.e., its active site is exposed at the external surface of the membrane (29)), and its activity is regulated by the composition and microviscosity of the external half of the bilayer (30). The temperature dependence of the fluorescence anisotropy parameter and the 5'-nucleotidase activity was determined between 4-40°C, and the logarithm of these parameters was plotted against 1/T (°K) to detect thermotropic transition. The breakpoint temperatures of the Arrhenius plots in the models investigated are reported in Table 2. These temperatures were higher in membranes from old, AL-fed animals than in those from young, AL and old, EOD animals, which were very similar.

It must be pointed out that breakpoints in the Arrhenius plots are closely associated with lipid phase separation processes occurring in the membrane, and that these temperatures are closely related to the membrane lipid composition (31). When comparing EOD- and AL-fed rats, the difference was in the order of 3°C, which cannot be compensated by an average body-temperature decrease of 0.9°C, occurring in undernourished animals

Table 2. Breakpoints of Arrhenius plots (°C) of liver plasma membranes

Temperature (°C)	*Young*	*Old AL*	*Old EOD*
DPH fluorescence polarization	16.3±0.3	19.5±0.6	16.7±1.4
5'-Nucleotidase activity	25.1±0.6	28.0±0.7	25.7±0.4

(Data in table are from Pieri et al. (28), with the kind permission from Elsevier Science Ireland Ltd., Bay 15K Shannon Industrial Estate, Co. Clare, Ireland.)

(32).

As summarized in Table 3, the same positive effect of FR on the physical-chemical properties of membranes was also demonstrated in other organs (23,33-36). Although in most of these studies membrane microviscosity was measured at a fixed temperature, the reported differences between AL and FR groups were so large that they cannot be compensated by the decrease of average body temperature occurring in FR animals. It is important to stress that these results were obtained using animals of various strains and, that a dietary intervention different from the EOD schedule was applied. Thus, the maintaining of membranes in a proper functional state seems to be a well-defined modification induced by FR, regardless of the type of FR applied and animal strain used. Based on these data, it has been suggested that the lowering of body temperature in FR animals may play a significant role in determining membrane properties. Indeed, the change in body temperature in these animals may induce modification of membrane lipid composition, stimulating the cell to counteract the rigidifying effect of a lower body temperature. The membrane is therefore maintained in a proper functional state by a mechanism similar to that found in poikilotermic animals (37).

On the other hand, the existence of such a mechanism in homeotherms has also been demonstrated. As an example, the body temperature of genetically obese mice was observed lower than that of their lean littermates as a result of defective thermogenesis (38). When the body temperature was normalized by housing the animals at 34°C or by thyroid hormone treatment, all the physical-chemical parameters of the liver cell mem-

Table 3. Effect of age and FR on changes in membrane fluidity.

Rat Strain	*Diet*	*Membranes*	*Age*	*FR*	*References*
Fischer 344	60% calories	Spleen cells	Decrease	Increase	33
Fischer 344	60% calories	Microsomes (liver)	Decrease decrease	Small	23,36
Fischer 344	60% calories	Mitochondria (liver)	Decrease	Small	23,36
Charles River	40% food	Erythrocytes	Not determined	Increase	34
Fischer 344	60% calories	Synaptosomes	Decrease	No change	35

brane returned to values close to those observed in the lean, control mice having similar body temperatures. It is interesting to note that the properties of the membranes were evaluated using the breakpoints of the Arrhenius plots of both DPH fluorescence polarization and 5'-nucleotidase activity, as done in the experiments using EOD-fed animals (28).

Most studies on age-related membrane deterioration with an increased membrane rigidity claim increased cholesterol/phospholipid ratio with age a causal factor of membrane rigidity during senescence. However, differences in membrane cholesterol content do not account for the difference in membrane microviscosity when comparing FR and AL-fed animals. In fact, it has been clearly shown that FR markedly attenuates the effect of age on fluidity without modulating the cholesterol/phospholipid ratios of the same membranes (23, 35). However, as described below, FR selectively influences fatty acid composition in tissues as varied as liver (39-41), kidney (42), splenocytes, and bone marrow cells (43). Interesting enough, the unsaturation/saturation ratio of membrane fatty acids, a parameter that influences membrane microviscosity, was maintained by FR at high levels throughout the life span of these rats (40,41).

In addition to the chemical composition, another factor can strongly influence membrane microviscosity, namely, lipid domain peroxidation (22-26), and this has been the subject of several studies using FR animals.

Peroxidizability of Cellular Membranes

As pointed out above, the free radical theory of aging, originally proposed by Harman (6), suggested that aging results from the cumulative, deleterious effect of random free radical action arising from oxidative metabolism. This theory stimulated a number of studies aimed to verify that an increase of peroxidative stress occurs in aged organisms.

Other studies have attempted to assess rates of free radical-induced damage in FR animals, and the results seem to support Harman's hypothesis (6). Indeed, the early indication that FR modifies the extent of free radical damage stems from the observation that protein restriction decreased lipofuscin accumulation in the brain and heart of mice (44). Subsequent experiments confirmed these data (45) and, in addition, demonstrated that lipid peroxidation, as measured by malondialdheyde content in liver homogenate, was reduced in FR animals, compared to the control groups (46). Further support for the modulating effect of FR on membrane lipid peroxidation was reported in subcellular membranes of liver (39,47), heart (48), and erythrocytes (27). The general picture emerging from these studies shows that membranes of FR animals are better protected against damage caused by free radicals than those of AL-fed animals.

Regarding the membranes, at least two mechanisms may account for the positive effect of FR. One is the level of antioxidants protecting the membranes, which may be different during aging and under FR treatment. Studies using rats fed a vitamin E deficient diet, clearly show that this vitamin could powerfully protect membranes against peroxidative damage (49, 50). However, serum, as well as membrane vitamin E levels, are higher in AL-fed animals compared to their FR counterparts (39). Thus, differences in vitamin E levels do not give a plausible explanation for decreased membane peroxidizability in FR animals.

The possibility that undernutrition modifies the chemical composition of membranes, thereby attenuating their peroxidizability, is supported by recent results. Studies performed on liver mitochondrial and microsomal samples (41), have demonstrated that membrane levels of long-chain polyunsaturated fatty acids increased progressively, while membrane linoleic acid decreased steadily with age. FR resulted in a significant increase in the levels of essential fatty acids, while attenuating the levels of C22:4, C22:5 and C22:6. A similar action of FR has been reported in tissues as varied as plasma (51), kidney (42), splenocytes, and bone marrow cells (43). Thus, while the unsaturation/saturation ratio of membrane fatty acids was maintained, the peroxidizability of the membrane was decreased because of the decrease in long-chain fatty acids having multiple, double bonds.

Studies on Mitochondria

Although it has been known for several years that respiring mitochondria produce free radicals (52, 53), there are few data concerning the relation among FR, mitochondrial free radical production, and peroxidative damage. In addition to the chemical composition of the membrane, at least two other factors may determine the rate of the peroxidative damage in living cells, namely, the amount of free radicals produced and the scavenging capacity of the antioxidant defense system.

Regarding the amount of free radical production, mitochondria in general, and the electron transport system in particular, are widely recognized as significant sources of oxygen radicals implicated in aging (54). However, some data support the notion that FR decreases the rate of free radical production in living cells. To the best of our knowledge, only one report shows that FR is able to prevent the age-dependent increase of free radical production in mitochondria isolated from brain, heart, and kidney of mice (55). The other data obtained so far refer to the respiratory control of isolated organelles (56) or deal with electron microscopic examination (57). However, a recent review shows evidence that free radical detoxification capacities of the cell are increased by FR (58).

This evidence is based on the activity of single components of the antioxidant defense system, and therefore represents a steady state level. However, one must

take into account that the synthesis of some antioxidants is necessarily induced during particular cell functions. As an example, reduced glutathione (GSH), a fundamental component of the antioxidant system, is synthesized and its level is almost doubled during cell proliferation (59). In addition, the antioxidant defense system should be considered as an integrated network in which a single component may substitute or compensate for another; thus, the determination of a single component of this system hardly accounts for the total activity.

Considering this finding, a new physiological model to investigate the peroxidative damage (60) and the eventual protection elicited by FR has been proposed: the in situ measurement of mitochondrial membrane potential during the proliferation of splenic lymphocytes induced by Concanavalin A (Con A). The rationale for using this model raised from experiments on isolated mitochondria and was based on the following: (i) increased respiration occurs during proliferation (61), which increases the risk of free radical production (62), especially in old cells (63); (ii) free radicals are produced during the mitochondrial electron transfer chain activity and the rate of superoxide radical formation is proportional to the rate of mitochondrial oxygen utilization (64); (iii) the activation of lipid peroxidation, regardless of the nature of prooxidant, causes an efflux of Ca2+ and other cations from mitochondria, a fall in membrane potential, and a swelling of organelles (65,66). Thus, by measuring the mitochondrial membrane potential, one can obtain information about the peroxidative stress that occurs during a fundamental physiological process, such as cell proliferation.

Mitochondrial membrane potential of splenocytes has been measured by flow cytometry after staining with the fluorescent vital probe Rhodamine-123 (Rh-123) (67). As shown in Fig. 1, the increased respiration occurring following mitogenic stimulation (68), results in an increase of mitochondrial polarization (high Rh-123 uptake) in the majority of splenocytes from young animals. In old rats, after an initial (24 hr from Con A stimulation) increase of the number of cells with highly polarized organelles, a depolarization of mitochondria (low Rh-123 uptake) occurred in about half of the cells, indicating that peroxidation of mitochondrial membrane occurred subsequent to the increased respiration. FR greatly influenced the distribution in the different cell populations. It increased the number of splenocytes with high Rh-123 uptake, even in the adult animals. Furthermore, FR allowed an increasing number of cells from old animals to maintain highly polarized mitochondria during the 3-day culture period (69).

Several studies were also performed to prove that the membrane potential of mitochondria, as measured by Rh-123 uptake, was sensitive to peroxidative stress in living cells. For this purpose, cells from vitamin E deficient animals (60) and normal splenocytes, supplemented (70) or depleted of GSH (71), in vitro, were investigated.

The main results of these studies were that the cells from vitamin E deficient animals, as well as those depleted of GSH, behaved like the cells from old, AL-fed animals. Indeed, the number of cells with depolarized mitochondria increased, while the number of cells with highly polarized organelles decreased, dependent on time of culturing. The impairment observed in cells from both vitamin E deficient and old, AL-fed animals was completely prevented by GSH supplementation of the culture medium (59, 72). From these data, it is safe to conclude that the membrane potential of mitochondria during proliferation is a sensitive parameter with which to monitor cellular peroxidative stress.

Results obtained using mitochondria from EOD-fed animals support the notion that mitochondrial membranes are well-protected during aging and/or that the amount of free radicals produced during mitochondrial respiration is lowered by FR, which is in agreement with Sohal et al. (55).

Although, beyond the scope of the present review, one must acknowledge that these data may have a profound impact on the mitochondrial hypothesis of aging (54, 73-76).

Concluding Remarks

Evidence is accumulating that FR preserves, among the other effects, the properties of the cellular membranes from the age-dependent modifications. These preservations may represent one of the ways by which the delay of the aging process in FR animals is realized. Regarding the mechanism, two concomitant effects may participate in the induction of this positive effect, as shown in Figure 2. The lowering of the body temperature that occurs in FR animals may stimulate the cells to build membranes with a composition that can counterbalance the rigidifying effect of lower temperature and maintain a proper membrane function through a more fluid state. In addition, the changes in the chemical composition of the membranes also decrease their peroxidizability, which together with the maintenance of protective enzyme activity (58), help to prevent the age-dependent deterioration of the cellular membrane.

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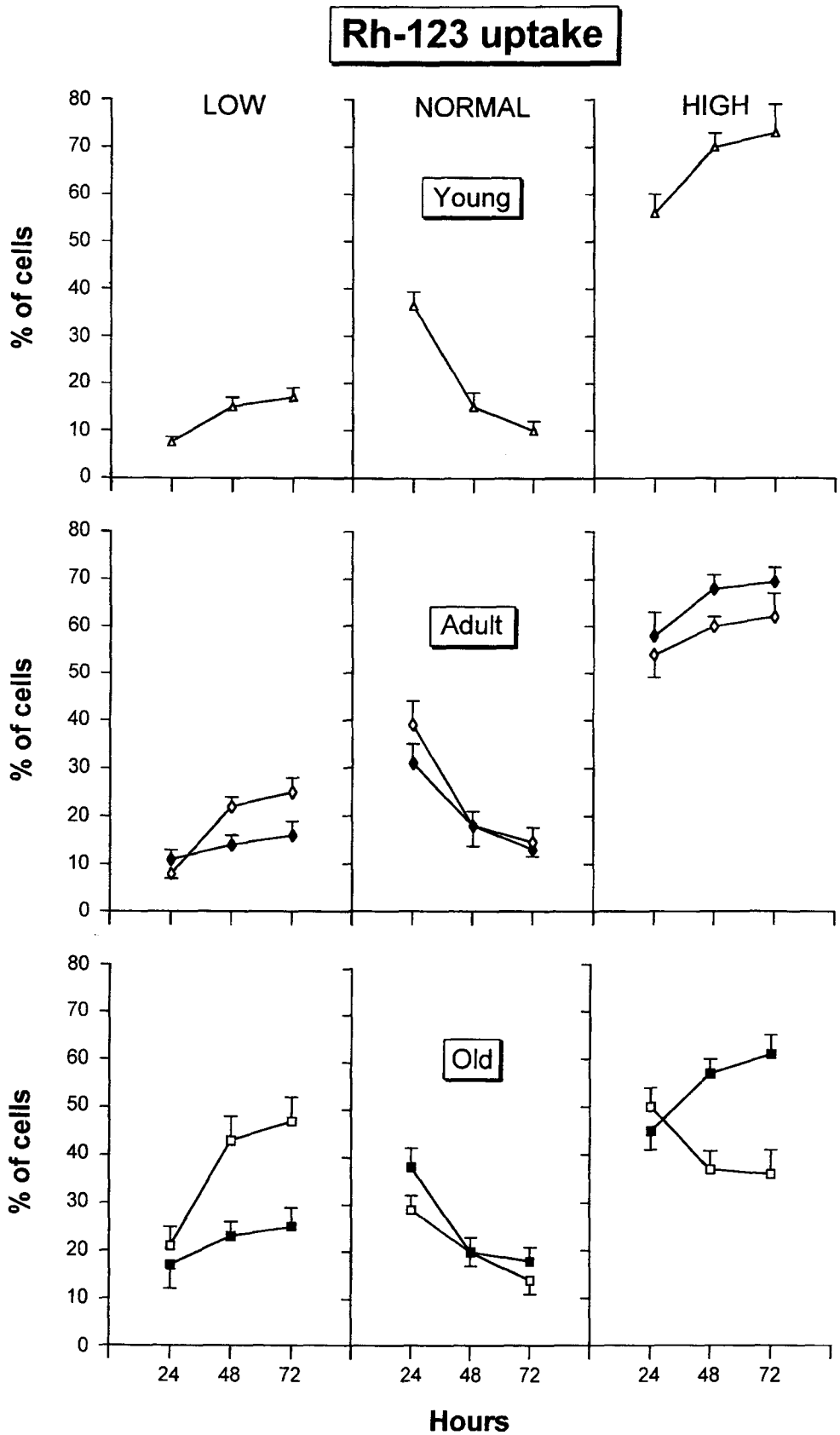

Figure 1: Time-dependent changes of splenocyte populations showing low, normal, and high Rh-123 fluorescence during lymphocyte proliferation. The symbols represent cells from young (□), adult (◇) and old (▫) AL fed rats, and adult (♦) and old (■) EOD rats. (Data are from Pieri et al. (66). Reprinted with the kind permission from Elsevier Science Ireland, Ltd. Bay 15K Shannon Industrial Estate, Co. Clare, Ireland.)

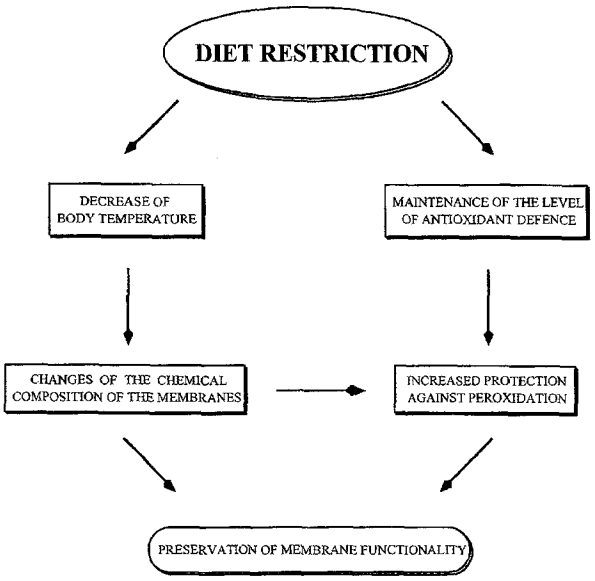

Figure 2: Suggested mechanism through which undernutrition prevents the age-dependent modification of cellular membranes.

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